Type III Secretion *à la Chlamydia*

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Abstract

Type III secretion (T3S) is a mechanism central to the biology of the *Chlamydiaceae* as well as many other pathogens whose virulence depends on the translocation of toxic effector proteins to cytosolic targets within infected eukaryotic cells. Biomathematical simulations, using a previously described model of contact-dependent, T3S-mediated chlamydial growth and late differentiation, suggest that chlamydiae contained in small non-fusogenic inclusions will persist. The model is further discussed in the context of *in vitro*-persistent, stress-induced aberrantly enlarged forms and of recent studies using small molecule inhibitors of T3S. A general mechanism whereby both early and mid-cycle T3S-mediated activities and late T3S inactivation upon detachment are critical for chlamydial intracellular development is emerging.

EB	elementary body
IB	initial/intermediate body
RB	reticulate body
mRB	aberrantly enlarged RB form induced by stress
Inclusion	parasitophorous vacuole that contains replicating chlamydiae
Injectisome	the T3S machine or “nano-syringe”
IM	inner (cytoplasmic) membrane
OM	outer membrane
T3S	type III secretion
vir-T3S	virulence associated T3S
fla-T3S	flagellar T3S
cds	“contact-dependent secretion” gene
cop	“*Chlamydia* outer protein” gene; ortholog of *yop*
scc	“Specific *Chlamydia* chaperone” gene; ortholog of *syc*
Tarp	translocated actin recruiting phosphoprotein

A family apart: The Chlamydiaceae

The *Chlamydiaceae* and other members of the order *Chlamydiales*[a] are obligate intracellular bacteria that infect a broad spectrum of multicellular organisms including human, animal and insect species, as well as unicellular organisms such as free-living amoeba. They are characterized by a unique biphasic developmental cycle that initiates when the infectious, metabolically inert elementary body (EB) attaches to and enters a eukaryotic host cell. Post-internalization, the EB differentiates into the non-infectious, but metabolically active reticulate body (RB), which replicates by binary fission for several generations within a parasitophorous vacuole, termed the inclusion. Growth and multiplication of the RBs continue for 18 to 36 hr, depending on the strain. Upon an unidentified late signal(s), RBs differentiate back to EBs via a poorly defined form, variably termed the initial or intermediate body (IB).

In addition to inducing their own internalization, chlamydiae interfere with host cell function including subversion of the cytoskeleton to facilitate intracellular redistribution of newly internalized EBs [1], early inhibition of apoptosis to ensure intracellular survival for the duration of the developmental cycle, and induction of cell death [2] to release chlamydial progeny upon completion of the cycle. The endpoint of chlamydial intracellular development is the release of newly made EBs associated with the death of the infected host cell thought to involve an apoptosis-like mechanism [3-5].

RBs that are subjected to stress (e.g. tryptophan starvation, exposure to antibiotics or phages) cease to divide although they continue to replicate [6,7],

yielding aberrantly enlarged, multinucleated forms that phenotypically resemble stress-induced filamentous forms of rod-shaped Gram-negative bacteria. Like other stressed bacteria, these aberrant RBs express elevated levels of stress-response proteins [8] and do not resume normal growth --and subsequent late differentiation in the case of chlamydial RBs-- until the stressor is removed. Although these forms are often referred to as persistent chlamydiae, a link with clinically "persistent" infection in humans is still unproven. Hence to avoid confusion and by analogy with Gram-negative filamentous bacteria (sometimes termed "maxicells"), stress-induced forms are referred to as maxi-RBs (mRBs) in this review.

The genetic intractability of *Chlamydia* has made direct functional analysis of suspected virulence factors prohibitively difficult. Moreover, experimental reproducibility often suffers from systematic contamination with "variant" chlamydiae and bacterial or eukaryotic debris as clonal isolation and purification of these obligate intracellular organisms is difficult. Because of these limitations, *Chlamydia* researchers have resorted to alternative approaches such as comparative analyses with better characterized systems, and the use of surrogate systems whenever possible. Comparative analysis of the highly conserved genomes --a "poor-man's" genetic system in *Chlamydia* research-- for instance, has led to the identification of tryptophan synthase as a key determinant of organ tropism in *C. trachomatis* infection [9].

We examine here the genetic organization and protein machinery of one of the better studied pathways of *Chlamydia* sp., the type III secretion (T3S)

system that mediates the translocation of bacterial toxins to the cytosol of infected cells in several important Gram negative bacterial pathogens. Based on early ultra structural observations, we have previously proposed the T3S-mediated contact-dependent hypothesis, whereby chlamydiae replicate strictly in contact with the inclusion membrane, while detachment and coupled T3S inactivation constitute the signal for late differentiation. The hypothesis is now expended through biomathematical simulations predicting persistence of chlamydiae under conditions where multiple inclusions are formed in a single cell, and discussed in the context of the chlamydial response to stress and inhibition of T3S.

Type III secretion of a different type

T3S, which facilitates the direct translocation of bacterial virulence factors to the cytosol of the target eukaryotic cell [10] (vir-T3S), has been described in major human pathogens such as *Yersinia*, *Salmonella*, *Shigella*, *Pseudomonas aeruginosa*, pathogenic *E. coli*, and the *Chlamydiaceae*, as well as in bacteria infecting plants [11,12]. Recently, vir-T3S genes have also been identified in the genomes of environmental *Chlamydia* species, *Candidatus* Protochlamydia acanthamoeba [13] and *Simkania negevensis* (Myers et al., unpublished), both of which grow in amoeba [13,14]. In some pathogens, e.g. *S. enterica* serovar Typhimurium (*S.* Typhimurium) [15] and *Y. enterocolitica* [16], multiple vir-T3S systems encoded within unlinked pathogenicity islands or plasmids have been described. Although the function of each individual vir-T3S

system is not clear, it is thought that the multiple systems are functionally distinct. The SPI-1 system of *Salmonella* for example is important for invasion of target cells [17], whereas the unlinked SPI-2 system is critical for intracellular growth and survival [15,18].

A genomic T3S pathogenicity "archipelago"

Across the *Chlamydiales,* genes encoding the structural proteins of the vir-T3S apparatus are found in three distinct conserved genomic clusters (Fig. 1) while genes encoding putative translocator proteins and flagellar-associated T3S (fla-T3S) proteins are at unlinked genomic sites in the *Chlamydiaceae,* and are apparently absent in the *Parachlamydiaceae*. The molecular G+C content of each chlamydial T3S cluster is close to 40%, similar to the rest of the genome and there are no apparent vestiges of recent integration events such as IS elements or repeats. This stands in sharp contrast with vir-T3S genes of other Gram-negative bacteria, whose clustering in chromosomal pathogenicity islands or on plasmids suggests that they have been acquired recently by horizontal gene transfer from a heterologous donor [19]. Figure 1 reveals that all *Chlamydia* sp. have conserved T3S clusters, both in gene content and genomic location, with the exception of two clusters of *C. trachomatis* and *C. muridarum* that are inverted relative to *ori*. A comparison of chlamydial T3S gene order with that of the T3S plasmid (pCD1) of their closest phylogenetic relative, *Y. pestis,* reveals loose similarities (Figure 1). It is however unclear whether T3S genes that appear to be missing in *Chlamydia* relative to *Yersinia* are truly missing or simply distantly related, hence unannotated as T3S homologs in genome

sequences. These differences may reflect a T3S injectisome that is functionally adapted to the developmental biology of *Chlamydia* and the need of this organism to survive host defenses on both sides of the eukaryotic plasma membrane.

What are chlamydial flagellar T3S genes for?

It is not currently known whether contemporary vir-T3S systems have evolved from an ancestral flagellar T3S (fla-T3S) system [20] or if both vir- and fla-T3S systems have evolved independently from a common ancestor [21]. *Chlamydia* represents a microcosm of this unresolved question as, in addition to vir-T3S genes, all members of the *Chlamydiaceae* examined to date, although they are non motile organisms, possess a subset of fla-T3S genes.

Several fla-T3S genes annotated as *flh*A, *fli*F, and *fli*I (homologs of *cds*V, *cds*J, and *cds*N) are present in two genomic clusters in all genomes of the *Chlamydiaceae.* These genes are not found in Protochlamydia or *Simkania* suggesting that their selective acquisition by the *Chlamydiaceae* –or loss by the *Parachlamydiaceae*-- may have played a role in the transition of ancestral chlamydiae from unicellular to multicellular hosts. FlhA, an essential component of the flagellar export apparatus, is normally housed within the FliF basal-body MS (membrane and supramembrane) Ring, where it also interacts with the flagellar ATPase FliI and its specific inhibitor protein FliH [22]. *fliA*, encoding Sigma-28 involved in flagellar gene transcriptional regulation in other bacteria, is immediately downstream of *flh*A in all chlamydial genomes. Although microarray and proteomic experiments have indicated that these genes are expressed at

mid cycle [23], their function remains a mystery as they potentially encode only a portion of the flagellar basal body. Do these genes encode a simplified form of a flagellum that provides motility within the inclusion? If so, what other gene products compose the putative flagellum? Are the chlamydial flagellar proteins able to interact with the vir-T3S injectisome, i.e. do they represent a reductive evolution solution to the need for multifunctional T3S systems? Or do these genes have an entirely novel function which cannot be inferred from sequence similarity with other systems?

The chlamydial T3S machine

All chlamydial genomes encode multiple conserved proteins of the vir-T3S injectisome, a molecular "nano-syringe" made of about 20-25 proteins, the translocator apparatus, and chaperone subclasses, which together are required for the assembly and functioning of the T3S pathway [24]. Conserved components of the chlamydial T3S machine are succinctly described in Table 1 and represented graphically in Figure 2.

The chlamydial injectisome

The predicted lipoprotein, CdsJ, is predicted to span the periplasmic space and associate with integral membrane proteins CdsR-V. CdsJ also likely interacts with the inner membrane protein CdsD that has also been detected in at the surface of *C. trachomatis* EBs (ORF664 in Tanzer *et al* [25]). The highly conserved N-terminus of CdsV displays seven predicted transmembrane domains, while the large C-terminus region is less conserved, more hydrophilic

and is predicted to be localized in the cytoplasm where it may interact with effector proteins, chaperones or other T3S apparatus proteins. The outer membrane ring of the injectisome, which is necessary for the T3S needle to cross the outer membrane, is composed of CdsC, a homolog of YscC of *Yersinia*. In other systems, the ring forms hexameric structures similar to the "rosette-like" structures observed by Matsumoto [26]. The inner diameter of the chlamydial rosettes, estimated at 4-5 nm, is similar to the inner diameter of the *Yersinia* outer ring at 4.5 nm [27] and close to that of the *Salmonella* outer ring at 7nm [28].

The translocator proteins

CopB, and its paralog CopB2 of *C. trachomatis*, are homologs of the *Yersinia* T3S translocator protein YopB and as such, are predicted to act as the entry point for the T3S needle and to facilitate translocation of secreted effectors across the plasma membrane of the eukaryotic host cell. CopB is detectable in the inclusion membrane after infection, consistent with its presumed function as a T3S translocator. In contrast, CopB2 is detected in the host cell cytosol [29], possibly reflecting a function for chlamydial translocator proteins distinct from that in other species, where only one copy of the gene is present. Similar to enteric T3S, a single CopB homolog is also found in Protochlamydia.

Effector proteins

In contrast to apparatus components, T3S effectors display little sequence homology although they often display common structural features and have similar enzymatic activities across bacterial genera. In other systems, effectors

harbor a variety of toxic effects ranging from cytoskeletal alterations, subversion of signal-transduction pathways, repression or activation of apoptosis, and may also disrupt host transcriptional regulation [12,30]. By analogy with other systems, effector proteins may be either secreted into the inclusion lumen, to potentially attack the host cell via receptors at the surface of the inclusion membrane, are deposited in the inclusion membrane, or are translocated directly to the cytosol of the host cell.

Attempts to identify T3S effector proteins of the *Chlamydiaceae* have had varied success. Supportive evidence may include direct sequence or secondary structure similarity with T3S effectors of other species and, possibly, linkage to or co-precipitation with other T3S orthologs [29,31]. A more reliable indicator is the demonstration of T3S-mediated secretion or translocation of a candidate effector by a surrogate host bacterium [29,32-35]. An extension of this strategy to the testing of T3S-dependent secretion by *S. flexneri* of hybrid proteins composed of a predicted T3S signal sequence fused to adenylate cyclase identified 24 new candidate effectors [36]. However, this method also identified several proteins that are not known and/or not likely to be secreted, including orthologs of FliH (CPn0859), a predicted arginine decarboxylase (CPn1032) and a beta-lactamase-like metal-dependent hydrolase (CPn0879) (Mark Pallen, personal communication). Notwithstanding the questionable reliability of surrogate T3S systems, several candidate effectors have been identified, the most prominent of which are briefly discussed below.

Tarp

The Tir-like effector protein Tarp is translocated via T3S by *Y. pseudotuberculosis* and is involved in the recruitment of actin to the *C. trachomatis* inclusion [35]. Like Tir, the translocated receptor for entero-pathogenic *E. coli* [37], Tarp may function as a receptor for an unidentified chlamydial intimin analog. Since the T3S machine is functional early [38], it is conceivable that Tarp is "preloaded" in the T3S needle of the EB so as to mediate early cytoskeletal changes during internalization [39]. In addition, Tarp-mediated actin recruitment may be to build a cytosolic "track" for *Chlamydia*-ladden inclusions. Tarp is activated upon tyrosine-phosphorylation by an unidentified eukaryotic kinase [40], as is the case for many other secreted virulence factors [41]. Likewise, Tir is phosphorylated by the protein kinase Fyn [42] a member of the Src kinase family [41]. The existence of a Src consensus motif in Tarp strongly supports that it is also phosphorylated by a Src kinase.

Inc proteins

Chlamydial T3S effectors include inclusion membrane proteins IncA, IncB, and IncC, whose *C. pneumoniae* orthologs are secreted by the *S. flexneri* T3S system [33]. IncC of *C. trachomatis* is also demonstrably translocated into the cytosol of HeLa cells by the *Y. enterocolitica* T3S system [38]. IncA is located on the outer face of the inclusion membrane toward the cytosol and is involved in the homotypic fusion of multiple inclusions of *C. trachomatis* [43,44] but not of sphingomyelin-containing vesicles [45]. IncA also forms long fibers extending from the inclusion that are used as cytosolic tracks mediating the formation of

secondary inclusions [46]. Transfection of *incA* into eukaryotic cells blocks normal chlamydial development in these cells [47,48].

CopN

A homolog of the *Yersinia* T3S regulator YopN, CopN is translocated in a T3S-dependent manner by *Y. enterocolitica* [29,32] and *S.* Typhimurium (SPI-1) [34]. Demonstrated late *cop*N expression [49,50] is consistent with CopN being involved both in T3S down regulation and physical shutoff of the injectisome as RBs start differentiating into IBs. YopN similarly is thought to block the T3S channel through a conformation-dependent interaction with its chaperone and a cytoplasmic membrane site of the T3S injectisome [51]. Contact with a susceptible cell (or removal of calcium *in vitro*) is presumed to disrupt this interaction, allowing YopN secretion and subsequent unblocking of the channel for other Yops. A direct comparison between YopN and CopN is not necessarily justified in view of the phylogenetic distance and biological disparities between *Chlamydia* and *Yersinia*. However, it is worth noting that similar roles for CopN and YopN are still possible since late expressed chlamydial proteins are likely to mediate early events in chlamydial pathogenesis.

The T3S contact-dependent development hypothesis

***Chlamydia* in the pre-omic era**

Starting in 1973, nearly 20 years before the earliest description of the T3S system in *Yersinia* [52], Matsumoto and colleagues published electron micrographs showing rosette-like structures and projections at the surface of

Chlamydia psittaci strain Mn [26,53-55]. These and similar structures have since been observed at the surface of *C. trachomatis* [56], *C. caviae* [57], *C. muridarum* [58], *C. pneumoniae* [59] EBs and have become known as Matsumoto's projections. In a few of the published micrographs, Matsumoto was able to demonstrate that the projections were anchored in the cytoplasmic membrane, extended through the outer membrane of the chlamydiae (Figure 3B), and that a cluster of hexagonally-arrayed projections delineated a zone of contact between the bacterium and the plasma membrane-derived inclusion membrane (Figure 3). Based on their analogies with T3S systems, we have previously proposed that Matsumoto's projections were in fact T3S injectisomes [60]. This hypothesis, while still begging for immunochemical, biochemical or genetic confirmation, is consistent with physiological and structural properties of T3S injectisomes. However, any model for chlamydial development that is built on the identity of the T3S machinery and Matsumoto's projections, including that developed in the paragraphs below, must be preceded by the caution that it remains an unverified hypothesis.

The size of the projections' patch decreases during development

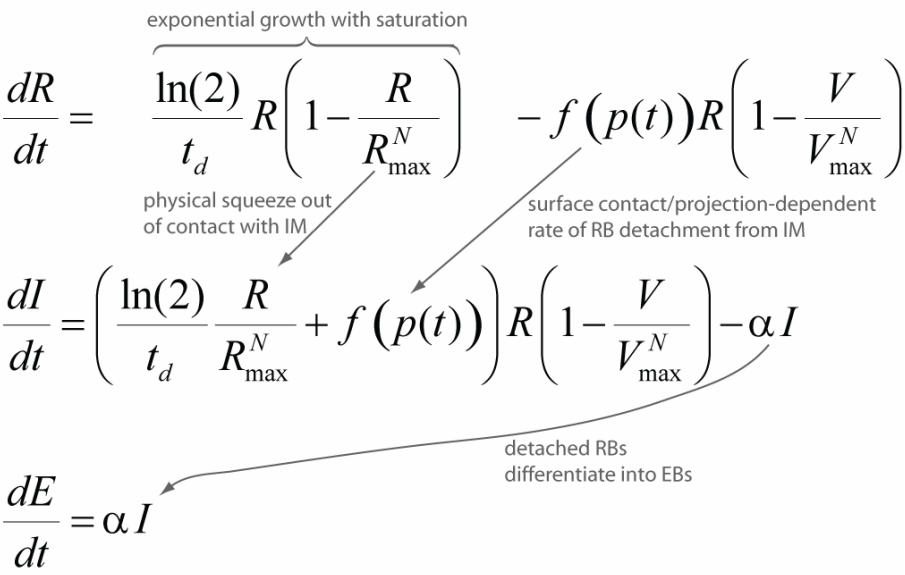
Chlamydial surface projections are organized as a regular hexagonally arrayed patch located at one pole of both developmental forms of the bacterium. Matsumoto observed that within a patch of *C. psittaci* strain Mn, the number of projections may vary from as low as 11 in the smaller patch of the EBs to as many as 83 in the larger patch of the RBs [54]. We previously hypothesized that the patch of projections observed on individual chlamydiae represents the fixed,

imprinted memory of the contact area between the chlamydial surface and the inclusion membrane and that “fixation” might occur late upon general oxidation of surface disulfide bonds [60]. Moreover, we speculated that the decrease in the number of projections during late differentiation signified that the contact area was being reduced as the number of replicating chlamydiae progressively exceeded available space at the inclusion membrane surface and were being physically “squeezed out”. An implication of this was that detachment from the inclusion membrane could represent the signal for late differentiation [60,61], echoing a previous suggestion by Hackstadt [62]. The proposed identity of T3S injectisomes and surface projections added a new dimension to this hypothesis in that it implied that T3S played an essential role in sustaining replication and conversely, that loss of T3S activity through loss of contact could be the signal for late differentiation. The presence of T3S projections on the EB further suggested a potential role for T3S, possibly through preloaded effectors, during the initial steps of infection.

Box 1: A biomathematical model of chlamydial development

Can mathematics succeed where genetics has so far failed? The T3S contact-dependent hypothesis was analyzed through biomathematical modeling [63]. Modeling the hypothesis formalizes its assumptions, quantifies it in alignment with experimental observations, and produces testable predictions of its implications. Biomathematical modeling can produce outcomes that are the logical conclusion of a number of given assumptions; the outcomes may be beyond first-level intuition but can be explained by the mechanistic model

components and in terms of the underlying assumptions in the equations. The current hypothesized biological model can be expressed mathematically by three differential equations, representing the rate of change in the number of RBs (*R*), IBs (*I*), and EBs (*E*) over time along the developmental cycle:



These equations take into account parameters that are either known or confidently estimated, such as the volume of space occupied by detached chlamydiae in an inclusion (*V*), the average number of projections post-infection derived from Matsumoto's observations (*p*(*t*)), and the doubling time of RBs during exponential growth (t_d). The model has been useful to verify the plausibility of the T3S contact-dependent hypothesis and to predict the testable implication of persistence according to constrained geometry. Once more experimental data becomes available, the model can be further fine-tuned and more complexity introduced.

Box 2: Utility and limitations of biomathematical modeling

A specific limitation of biomathematical modelling owes to the quality and quantity of available measurements used to inform the development of model equations and parameters. In *Chlamydia* research, this is a significant hurdle as the organism presents an unusual degree of experimental difficulty. For example, the number of projections over developmental time has only been measured once in a single *Chlamydia* species [54]. This result was obtained long before T3S was discovered and laid nearly forgotten for 20 years. Matsumoto's data has been used as an estimate of projection numbers in the model, and the model is calibrated to this data so that it produces normal developmental time courses similar to observations. Future improved measurements will continuously test the model and allow its refinement. This in turn will provide a quantitative framework against which other measurements can in turn be tested.

This model is also deterministic in nature, not accounting for general stochasticity, especially for very small numbers of particles. Future models could incorporate greater geometrical features so that more specific predictions can be made. With any mathematical model, care must be taken with the assumptions that underlie it and they should be made explicit. If an important assumption used in a model is incorrect, then all subsequent model outcomes will be biased accordingly. The current model explicitly makes certain assumptions (such as RBs replicate only in contact with the inclusion membrane and they detach from the membrane once the number of projections decreases to a threshold level). The mathematical model is adequate to explore the outcomes of these

assumptions and the model results are, by nature, implications of this hypothesis. The model is not valid outside these assumptions.

Mathematical modelling is an under-exploited area of biology. Productive application of mathematics to biological systems should be evaluated on a case-per-case basis. But the *Chlamydia* system lends itself exquisitely to modelling because it is a "closed" system, i.e. most of chlamydial biology occurs within the physically restricted, measurable space of the infected cell. A highly significant asset of the chlamydial model is that "simulations" that mimic real biological events can be produced easily; for example, the cases of *in vitro* persistent aberrantly enlarged mRBs and multiple inclusions are simulated by altering input parameters and re-running the computerized model. Mathematical modeling allows *in silico* experiments to be run and re-run with different parameters at no real costs, whereas wet lab experiments are expensive and time-consuming. Modeling can predict specific relationships and threshold levels critical for development and can determine sensitivity relationships between outcomes and experimental conditions. Experimentation can produce results that modeling can attempt to describe and explain, and then modeling can inform experimental design and provide experimentally testable predictions. As has occurred in physics for many decades, experimentation and modeling theory are highly compatible and complimentary disciplines, but their union in biological applications is currently under-developed and could be utilized considerably more.

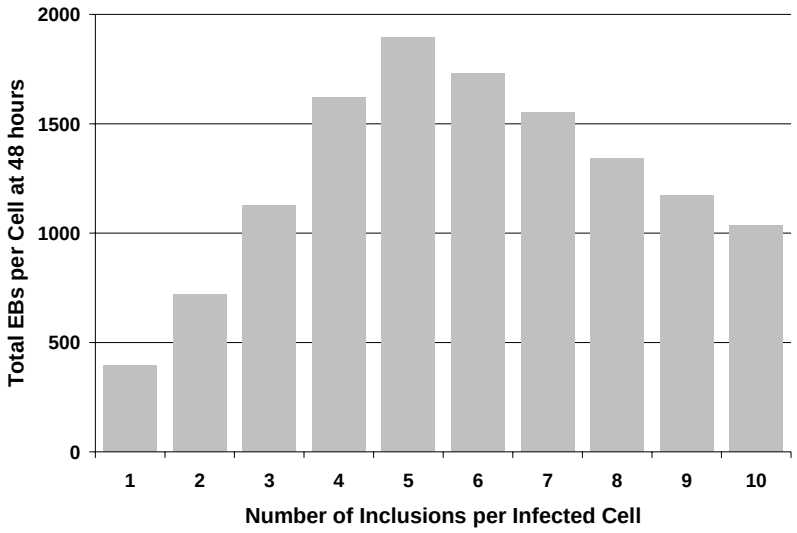
T3S mediates intracellular development

The involvement of chlamydial T3S in growth and development is a departure from the role of T3S in other pathogens where T3S is indispensable for infection but dispensable for growth. This hypothesis for modulation of growth has been shown to be consistent with observations and preliminary mathematical modeling analyses [63]. We now revisit and extend the model to encompass two alternate outcomes of chlamydial growth: the development of inclusions that persist *in vitro* and that of multiple inclusions. Essential steps and presumed effector involvement are represented graphically in Figure 4.

The strength of the model is that it reconciles many old and new observations and provides a unified concept of chlamydial growth and differentiation across species boundaries. For instance, progressive detachment from the inclusion membrane is consistent with the perennially observed lack of developmental synchronicity such that EBs, IBs and RBs coexist in the late inclusion, even after careful synchronization of the initial infection step. The model however is also noteworthy in that it goes against the commonly held belief that chlamydiae are able to replicate free in the lumen of the inclusion, i.e. out of contact with the inclusion membrane. This belief is based on observation accumulated during nearly 40 years of growing chlamydial inclusions by transmission electron microscopy (TEM). Indeed, TEM which reproducibly generates high contrast images also reproducibly introduces systematic "displacement" artifacts owing to the harsh dehydration conditions, subsequent embedding in a resin and thin-sectioning.

Box 3: Are small, non-fusogenic inclusions persistent?

The number of inclusions per infected cell varies between species and within species. Multiple inclusions within a cell may arise from initial infection by multiple EBs or by inclusion division. Conversely, multiple inclusions within a cell may fuse to form a single late inclusion. In *C. trachomatis*, inclusion fusogenicity is modulated by the type III secreted protein IncA, whereby inclusions containing mutant *incA* do not fuse [43]. Biomathematical simulations indicate that varying the number (*N*) of inclusions per cell increases EB progeny up to *N*≈5, and decreases thereafter (below).



This is a logical result: as the number of inclusions increases, there is increased RB surface area in contact with the inclusion membrane and this facilitates greater growth overall until space becomes restrictive (only once there are ~5 inclusions). Increased numbers of inclusions implies that the size of each inclusion decreases so that available luminal volume for detached RBs is

restricted. Therefore, the T3S contact-dependent model predicts that chlamydial species that tend to produce multiple inclusions will be characterized by RBs that do not detach from the inclusion membrane but “persist” as RBs. Although this has not been investigated systematically, it is well-known that chlamydial species that produce multiple or lobar inclusions (e.g. *C. pneumoniae* and many veterinary *Chlamydia* species) tend to grow “forever” *in vitro* and/or are characterized by inclusions tightly packed with RBs devoid of luminal space. Non-fusogenic strains of *C. trachomatis* are also more frequently associated with sub-clinical, “persistent” infection than are their fusogenic counterparts [64].

In vitro-persistence is a “consequence” of the model

The phenomenon known as *in vitro*-persistence, which results from exposing chlamydiae to stress, provides strong indirect support to the model as well as to the predicted persistence of small inclusions (see Box 3). Stressed mRBs are aberrantly enlarged, typically reside within relatively small inclusions and are therefore characterized by the same physical constraints as normal-sized, ‘persisting’ RBs in multiple inclusions, albeit on a different spatial scale.

Whether RBs (or mRBs) are attached or detached from the inclusion membrane is directly linked to whether T3S-mediated translocation of effectors to the host cytosol is on or off. This leads to the hypothesis that T3S turn-off on its own (i.e. with or without detachment) may be the signal for late differentiation and that detachment merely facilitates the process. Comparative transcriptomics of normal vs. IFN-gamma induced in vitro-persistent *C. trachomatis* indicate that

transcription of T3S genes is unaffected [23], suggesting that mRBs continue expressing and assembling T3S injectisomes at near normal rates. The net result should be more T3S injectisomes per mRB (compared to normal RBs), contributing to tether the mRB to the inclusion membrane. The T3S effector CopN is not expressed in these cells and is only expressed late in normal RBs, coincidental with the expression of the cysteine-rich outer membrane protein OmcB, i.e. at the developmental stage where the first late developmental forms are usually observed. A chicken-and-egg question then becomes: Does CopN provoke RB detachment and coupled T3S shut-off, or is CopN expression a consequence thereof? Recent evidence for cytosolic targets of CopN in late infected cells (Peters & Bavoil, unpublished) favors the former mechanism, albeit tenuously.

Small chemical inhibitors of T3S inhibit development

Small chemical compounds that belong to a class of acylated hydrazones of salicylaldehydes have been recently shown to specifically inhibit T3S and to significantly alter development as demonstrated by a dramatic reduction in the infectious EB yield [65-67]. One such compound, INP0010, coincidentally inhibited T3S and development in *C. pneumoniae* infected cells [67]. Two other compounds, C1 [65] and INP0400 [66] were able to block T3S-mediated secretion of IncA of *C. trachomatis*, resulting in the inhibition of homotypic vesicle fusion and formation of multiple small inclusions. INP0400 inhibition, but not C1 inhibition, was coupled with the detachment of RBs from the inclusion

membrane. An important question then becomes how to reconcile these findings with our prediction that T3S inactivation provokes late differentiation.

A major difference between the predicted late T3S inactivation upon detachment and inhibition with small chemical compounds is in the timing. In our model T3S inactivation occurs “naturally” upon detachment, i.e. presumably after all T3S effectors have been secreted. Chemical inhibition applied at the onset of infection in contrast may target T3S-mediated secretion of early and mid-cycle effectors, which are necessary for growth, further development, and presumably further T3S. The observation that compound C1 added late (15 hours post-infection) still inhibits *C. trachomatis* development [65] suggests that this inhibitor may alter secretion of a late effector. It is also intriguing that different inhibitors appear to have opposed activities on systems that are very similar. For instance, INP0010 inhibits *C. pneumoniae* T3S, has no effect on T3S of the closely related *C. trachomatis*, but blocks that of the more distant *Yersinia pseudotuberculosis* [67]. This is indicative of a high level of specificity that suggests that secretion of a specific effector(s), or the effector itself, is the target of the inhibition. The contact-dependent hypothesis developed here and recent results of small inhibitor studies are overall consistent with a model proposed by Wolf and collaborators whereby signals governing development are transduced back through the T3S apparatus [65] by a specific mid-cycle to late effector. Failure to transduce these signals or failure to secrete the effector would down-regulate late T3S expression and/or function resulting in a developmental block. Although inhibitors studies are still in their infancy and the actual targets of these inhibitors

are not known, they hold the potential for unraveling some of the most intricate aspects of chlamydial biology while at the same time providing new avenues for therapeutic intervention.

Conclusion

The presence of T3S genes in all *Chlamydia* species examined to date suggests that T3S is essential to the survival of these bacteria. This is a fundamental difference with other pathogens in which T3S may be optionally present or where it can be inactivated by mutation without penalty to the bacterium. A mathematical model based on the strict replication of chlamydiae in T3S-mediated contact with the inclusion membrane, predicts that loss of contact and coupled T3S inactivation constitutes the signal for late differentiation. Biomathematical simulations suggest that chlamydiae contained in multiple small inclusions will persist as do stress-induced aberrantly enlarged mRBs. Although this simple model is applicable to *Chlamydia* across species boundaries, future refinements based on new findings, e.g. differential activities of small molecule inhibitors of T3S in different species, will introduce complexity reflecting specific host-pathogen interactions that can be further simulated biomathematically. Notwithstanding predictable variations on the theme, the model and observations reported above suggest that the fundamental role of T3S in the success of *Chlamydia* as a parasite may be to modulate its efficient growth and development inside the host, a role that may overshadow its presumed role in virulence.

Footnote:

[a]The "compromise" *Chlamydiaceae* taxonomy of Kalayoglu and Byrne (http://141.150.157.117:8080/prokPUB/chaprender/jsp/showchap.jsp?chapnum=335) is used in this article.

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Tables and Figures

Table 1. Components of the chlamydial T3S machine and secreted effectors. Fla-T3S homologs present in *Chlamydia* are in bold.

Figure 1: Multiple T3S gene clusters in the *Chlamydiales*. T3S gene clusters of *C. trachomatis* [68], *C. caviae* [69], *Candidatus* P. amoebophila [13] and *S. negevensis* (Myers *et al.*, not shown) (see legend to Fig. 1) are displayed using the *cds*N cluster as a reference. The pCD1 T3S gene cluster of *Y. pestis* is shown for comparison. Gene names and ORF numbers are listed above and below each gene when available at the TIGR Comprehensive Microbial Resource database (www.tigr.org/). Not drawn to scale.

Figure 2: Diagram of the chlamydial T3S machine. The putative structure of the chlamydial injectisome is derived by comparison with the *Yersinia* vir-T3S and *Salmonella* fla-T3S apparati (http://www.genome.jp/kegg/) [24]. Stipples identify components for which a paralogous fla-T3S protein (in parentheses) is found in the *Chlamydiaceae*.

Figure 3: Details of the RB interaction with the chlamydial inclusion membrane. HeLa 229 cells infected with *C. caviae* GPIC were examined by scanning (A) and transmission (B) electron microscopy. T3S projections (red arrows) are viewed from the cytosolic side of the infected cell extending across

the inclusion membrane from underlying RBs (A), or in cross-section of an RB bound to the inclusion membrane (B) with needle-like structures. In (B), the patch of projections delineates the area of contact between the RB and the luminal face of the inclusion membrane. (Courtesy of Professor Akira Matsumoto).

Figure 4: Graphic representation of T3S-mediated chlamydial development. The diagram highlights the predicted dependence of key stages of chlamydial infection on a functional T3S system from internalization to the onset of late differentiation. These include the translocation of preloaded Tarp during internalization (left); the role of IncA, mutant IncA* or down-regulated IncA in inclusion fusogenicity (top); the increased area of contact (and consequent T3S activity) between stress-induced mRBs and the inclusion membrane (bottom); and the coincidental expression and secretion of CopN during late differentiation (middle). The figure and time scales are approximate.